

One label, one tube, Sanger DNA sequencing in one and two lanes on a gel

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Sequencing in less than four lanes on a gel, using only one fluorescent label for the four bases (without variations in electrophoretic mobilities or problems with spectral overlap) in one tube was introduced in (1). In that process T7 DNA polymerase and buffer containing magnesium ions were used. By adjusting the ddNTPs ratios the sequence is deduced from the peak magnitudes corresponding to the four bases. The resolution was limited by nonuniformity of the peaks and by decrease of overall labelling due to the two-step process used in that sequencing protocol. In the method described in this note, applicable both to fluorescent and radioactive endlabelling, we have used the one-step protocol with manganese ions in one tube as described (2). This protocol results in uniform overall labelling (within 15% up to base 300). Biochemicals, annealing, termination and stop mixes, as well as diluted enzyme were as described in (4). Briefly, 1.25 μ g of M13 ssDNA in 5 μ l solution were combined with 2 μ l fluorescently labelled primer (2 μ M) and 6 μ l annealing mix. The mixture was denatured at 65°C for

3 minutes and cooled down to 25°C. Then 4U of T7 DNA polymerase in 2 μ l were added and mixed. Nucleotide mixes, as described below, were added and strand synthesis proceeded for 10 minutes at 37°C. The reaction was stopped with 20 μ l stop mix containing 10 mM NaOH for complete denaturation. Before loading, samples were heated at 65°C for 3 minutes and cooled down on ice. The amount of sample is sufficient for 4 gel runs on the EMBL automated fluorescent DNA Sequencer or the A.L.F. Sequencer (Pharmacia LKB).

One lane, one tube reaction: Nucleotide mixes were prepared from the termination mixes (4) in a ratio of T:C:G:A = 2:2:1:0.5 (6 μ l of T termination mix, 6 μ l of C-mix, 3 μ l of G-mix, 1.5 μ l of A-mix).

Two lane sequencing reactions: Nucleotide mixes were prepared as above, in a ratio of 1:1 for the T>C and 2:1 for the G>A reaction.

Fig. 1 (6) shows raw data obtained in the standard (2) four lanes one-step protocol (Fig. 1A), in two lanes (Fig. 1B) and

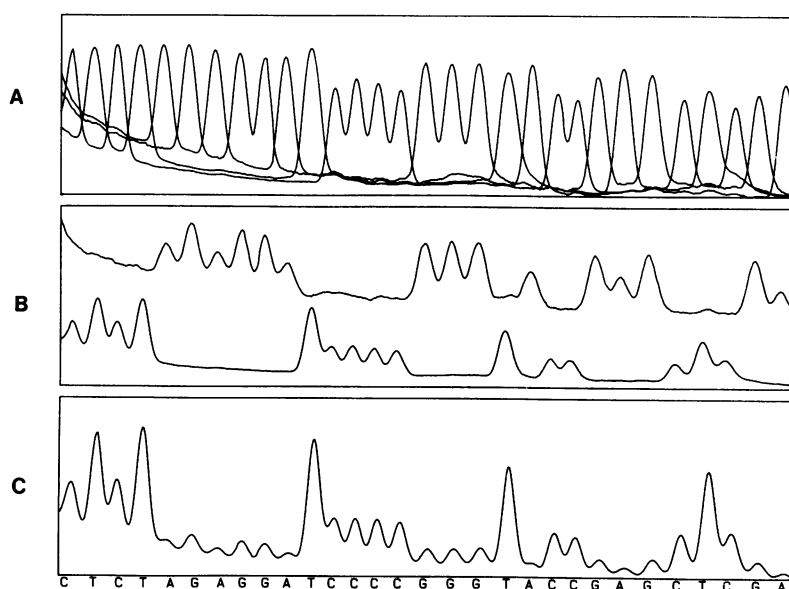


Figure 1.

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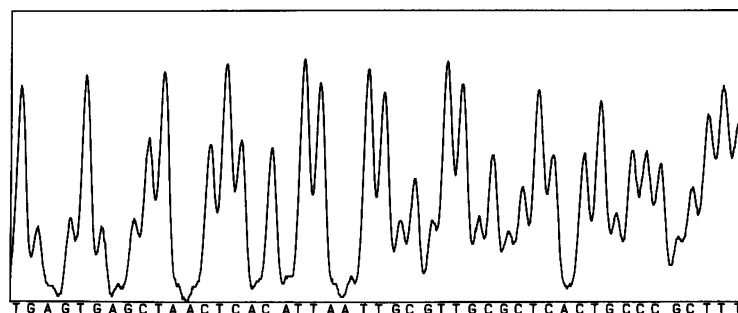


Figure 2.

one lane (Fig. 1C) according to the above protocols. Displayed are bases 22–53 of the M13mp18 DNA analysed on 6% gel with separation distance 20 cm (original in four colours). As shown in Fig. 2 (displayed bases 160–206), the resolution in the one lane method is at least 200 bases (as expected it is higher in two lanes, around 300 bases, data not shown). Using higher gel concentration (8–10%) or longer separation distance, we believe the resolution could be improved to around 300 bases or higher, the upper limit being given by resolution of single bases in the gel. We noticed different discrimination levels for the four ddNTPs by the T7 DNA polymerase in the presence of manganese ions. When equal molar ratios of the ddNTPs (1:1:1:1) are used in the one tube reaction, the resulting peaks are not of the same magnitude, but increase in the order $A < G < C < T$. This 'natural' preference of the T7 DNA polymerase is respected in the present protocol, in which the dideoxy molar ratios are changed slightly to obtain the desired magnitudes of peaks. The molarities can be possibly further optimised. The discrimination by T7 DNA polymerase of standard unlabelled ddNTPs, present in equimolar quantities during the extension, has not been reported previously. As in any method using only one lane for sequence analysis, the accuracy depends critically on template purity. Further parameters of importance may be the age of manganese solutions and content of salts in buffers. The accuracy is highest in the absence of secondary peaks, fullstops or high noisy background. At present this is best achieved by methods for purification of M13 ssDNA templates (3), with ⁷deaza-dGTP to eliminate compressions, where the error rate is below 1%. Direct sequencing with this protocol of plasmid or cosmid DNA, where the background may often be quite noisy, would result in higher error rate. As shown (4, 5), in these cases the four lanes method gives higher accuracy, since it is possible to follow each base lane independently and discern accurately peaks from the background. However, in the future purification methods for plasmids and cosmids will be further improved and result in low uniform background, making their sequencing in one or two lanes more accurate. The use of only one dye may be significant for the 'walking primer' strategy. At present we use this method for mapping of sequences, rapid screening of clones and for DNA sequencing in thin capillaries.

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6. Figure 1 and the principal of the method was presented at the Genome Sequencing Workshop, Wolf-Stradt, October 1987.